Netting Liver Disease: Neutrophil Extracellular Traps in the Initiation and Exacerbation of Liver Pathology

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Abstract

The liver plays a vital role in the immune system. Its unique position in the portal circulation and the architecture of the hepatic sinusoids, in combination with the wide-ranged population of immunocompetent cells, make the liver function as an immune filter. To aid in pathogen clearance, once challenged, the liver initiates the rapid recruitment of a wide variety of inflammatory cells, including neutrophils. These neutrophils, in conjunction with platelets, facilitate the release of neutrophil extracellular traps (NETs), which are web-like structures of decondensed nuclear DNA, histones, and neutrophil proteins. NETs function as both a physical and a chemical barrier, binding and killing pathogens circulating in the blood stream. In addition to their antimicrobial role, NETs also bind platelets, activate coagulation, and exacerbate host inflammatory response. This interplay between inflammation and coagulation drives microvascular occlusion, ischemia, and (sterile) damage in liver disease. Although direct clinical evidence of this interplay is scarce, preliminary studies indicate that NETs contribute to progression of liver disease and (thrombotic) complications. Here, we provide an overview of the pathological mechanisms of NETs in liver disease. In addition, we summarize clinical evidence for NETs in different disease etiologies and complications of liver disease and discuss the possible implications for the use of NETs as a diagnostic marker and a therapeutic target in liver disease.

Keywords
- liver disease
- coagulation
- neutrophil extracellular traps
- liver immunity

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Part I: Background: Liver Immunity and Neutrophil Extracellular Traps

The Liver: Center of the Immunological Universe

Although classically understood for its role in metabolism, lipid processing, and plasma protein production, the liver also represents a key element in the vertebrate immune system. Not only does the fetal liver serve as a primary site of hematopoiesis and lymphopoiesis in vertebrates,[1] the post-embryonic liver represents a key structure responsible for screening and filtering the blood of pathogens, for initiating and coordinating systemic immune responses and as a frontline battleground in the fight against viral and bacterial infections.[2]

Blood supply to the liver is provided by a confluence of oxygenated arterial blood (hepatic artery) and oxygen-depleted venous blood from the portal vein. It is this positioning within the portal circulation, located between the gut and the heart, that enables the liver to function as a critical immune sentinel. In fact, 80% of the blood supply to the liver is provided by the blood returning from the gut,[3] potentially carrying any pathogens or pathogen-derived molecules (pathogen-associated molecular patterns

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[PAMPs]) that may have entered the body through the gastrointestinal tract.

The unique blood vessel architecture of interconnecting sinusoids serves to slow the blood, maximizing the contact of circulating pathogens with the vessel walls and, importantly, intravascular leukocytes residing in the liver. In humans, this honeycombed structure of capillary-like vessels reduces blood flow by 50-fold as compared with arterial circulation,[4] slowing the passage of circulating pathogens, allowing time for the immune system to both detect and filter these entities from the blood. Despite this reduced blood flow in individual sinusoids, the size of the liver (the largest internal organ in the body[5]) and its overall structure ensure that 25 to 30% of the entire blood volume is filtered through this tissue every minute.[6,7] This places the liver at a key position in the body, functioning as an immune filter between the outside world (gut) and the rest of the body.

Within the liver parenchyma are a wide variety of both hematopoietic and stromal cells that have potent immune function. Cells lining the capillary-like sinusoids, the liver sinusoidal endothelial cells (LSECs), are capable of recognizing and binding pathogens and PAMPs.[8] LSECs express an array of complement, immunoglobulin, and scavenger receptors.[9] These molecules facilitate the capture and, under the right conditions, the internalization of circulating targets. In addition, LSECs also express several pathogen-recognition receptors, including several toll-like receptors (TLRs) that allow these cells to directly respond to PAMPs and to initiate a robust immune response.[8]

Below the layer of LSEC are hepatocytes, the principal parenchymal cells of the liver. Again, in addition to their widely appreciated role in metabolism, hepatocytes also contribute to the host immune response. Like LSEC, hepatocytes express a variety of receptors and are capable of internalizing pathogens.[10] Recognition of pathogens triggers an innate immune response, including cytokine production, that can help attenuate disease processes such as viral replication.[11] Additionally, hepatocytes present antigen to T cells helping drive the adaptive immune response.[12]

Beyond the immune capacity of the liver stromal cells, this tissue is also home to a variety of resident immune cells. Perhaps the best recognized and characterized of these leukocyte populations are the Kupffer cells (KCs). KCs are intravascular, nonmotile macrophages. Although much controversy surrounds our understanding of the origin of KCs, it is generally accepted that these cells are derived from a self-renewing liver stem cell.[13] KCs are located along the luminal wall of the liver sinusoids, ideally positioned to screen, sample, and filter the passing blood. These macrophages express a broad array of receptors (complement, immunoglobulin, scavenger, TLR) that facilitate the capture and clearance of circulating pathogens.[14] The central role these cells play in host defense is perhaps best exemplified by studies in which selective depletion has removed these macrophages from the liver.[15,16] In these studies, pathogen dissemination, through the blood, to other organs is readily observed and outcomes in models of infectious disease are greatly compromised. Upon detection of a pathogen, KCs are able to rapidly initiate a coordinate immune response, facilitating the recruitment of large numbers of neutrophils and monocytes, mediating antigen presentation to both cytotoxic T cells and helper T cells,[17] along with activation of invariant natural killer T cells and natural killer cells.[16] In many ways KCs are the principal coordinators of liver immunity as they possess the potential to shape the overall response into effective, protective immunity, or, in cases of disease, into a dysfunctional, pathogenic inflammation.

Despite the enormous potential to initiate and drive immunity in response to pathogens detected in the blood, the liver, under basal conditions, tends toward immune tolerance.[14] In this way, the liver avoids pathogenic responses to common food-derived antigens and maintains tolerance to baseline levels of PAMPs (e.g., lipopolysaccharide [LPS]) that may enter from the gut.[17] This creates a unique situation whereby balance between homeostasis and function immunity must be maintained. Failure to maintain this balance can lead to pathogenic inflammation to harmless stimuli, or a failure to respond to infectious agents, a scenario often exploited by hepatotropic viruses (e.g., hepatitis B virus).

Neutrophils and Neutrophil Extracellular Traps

Many liver disease etiologies are hallmarked by the presence of robust inflammatory cell recruitment to the liver vasculature. This inflammatory infiltrate is often dominated by the presence of large numbers of neutrophils. Neutrophils possess a diverse arsenal of antimicrobial effector mechanisms including phagocytosis, oxidative burst, degranulation, and the release of neutrophil extracellular traps (NETs). NETs comprise a mesh of decondensed nuclear DNA, which is released into the extracellular environment and decorated with a variety of both nuclear (histone, high mobility group box 1) and granular (myeloperoxidase [MPO], neutrophil elastase [NE], proteases) proteins.[18] These DNA structures take on a fibrous, web-like structure, expanding into the extracellular environment and often covering areas several times larger than the actual neutrophil. NETs allow the neutrophil to extend its reach, ensnaring and killing fast-moving microbes and sequestering pathogens, preventing their dissemination through the body. Although there is some disagreement regarding the specific process by which NETs are generated, the general acceptance is that multiple pathways exist by which NET release can be facilitated. Whereas lytic release, often referred to as NETosis, is frequently observed in vitro and has been shown to be dependent on oxidative burst,[19] a different mechanism has been observed in vivo whereby NETs are released from viable neutrophils and this process is not absolutely dependent on NADPH oxidase.[20] Where these models agree is on the need for histone modification, specifically citrullination, and on the requirement for the expression of peptidylarginine deiminase 4 (PAD4) by the neutrophil.[21] Animals deficient in PAD4, or treated with a PAD4 inhibitor, demonstrated dramatically reduced production of NETs.

NETs have been shown to interact with, bind, and capture bacteria, fungi, and viruses.[18,22,23] Once entangled, the various antimicrobial protein constituents of the NET work to kill/neutralize the pathogen. In this fashion, NETs function as

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both a physical (binding and holding the pathogen) and a chemical/molecular (proteases, histone) barrier. Importantly, these studies have shown that NETs can effectively limit bacterial dissemination and reduce viral infection of host target cells, augmenting the host innate immune response.[18,22,23]

In the liver, NETs have been shown to be induced by a variety of mechanisms including infection, ischemia, and sterile damage.[24] These web-like structures line the vessel wall of the sinusoids and have been shown to interact with several cell types, including the KC, neutrophils, and platelets, within the hepatic microenvironment. Although these extracellular DNA webs can contribute to pathogen clearance, they do so at some expense to the host. Largely attributable to their antimicrobial proteins, NETs are also strongly cytotoxic to nearby host cells.[25] This cytotoxicity has been shown to inflict collateral damage. Moreover, in addition to their cytotoxicity, NETs have also been shown to directly interact with the hemostatic system, triggering disseminated activation of hemostasis leading to vascular occlusion and further tissue damage.[26] These self-inflicted wounds contribute to organ dysfunction and the progression of several disease conditions, some of which we will address in the subsequent sections of this review.

Neutrophil Extracellular Traps, Platelets, and Coagulation

One of the most interesting and most complicated cellular relationships that exist with respect to NETs is that corresponding to the neutrophil–platelet–NET axis (►**Fig. 1**). Platelet–neutrophil interactions have been shown to be one of the key initiators of NET release.[27] Inhibition of these interactions have been shown to attenuate NET release in multiple models of both bacterial and viral infections.[28]

Studies have determined that integrin-mediated binding of platelets to the surface of adherent neutrophils under flow conditions can trigger DNA decondensation and release from the neutrophil.[29] These works indicate that a physical, shear-dependent mechanism is involved in the neutrophil activation steps upstream of NET release. In addition to integrin-mediated adherence, studies have also shown that platelet–neutrophil interactions can be facilitated by P-selectin (CD62P) on the platelet binding to constitutively expressed P-selectin glycoprotein ligand 1 (PSGL-1) on the neutrophil, though it is unclear if this selectin-mediated association can drive NET formation.[29] Other studies have indicated that a soluble component(s), released from the platelet,[30] can also contribute to neutrophil activation, although this soluble mediator pathway appears much less efficient at inducing NET release than does direct neutrophil–platelet interaction. This reduced efficiency of NET production suggests either the soluble factors are rapidly lost to/diluted in blood, or, these soluble factors have a very short half-life and are rapidly neutralized.

Once produced, NETs directly feedback onto the platelet, amplifying platelet aggregation and activation. Platelets have been shown to become trapped in NETs, binding to the extracellular DNA scaffold.[31] Destruction of the DNA backbone, using intravenous DNase, reduces platelet aggregation within the liver following bacterial infection.[32] Histones on the NET directly activate platelets through TLRs, linking the NET structure to

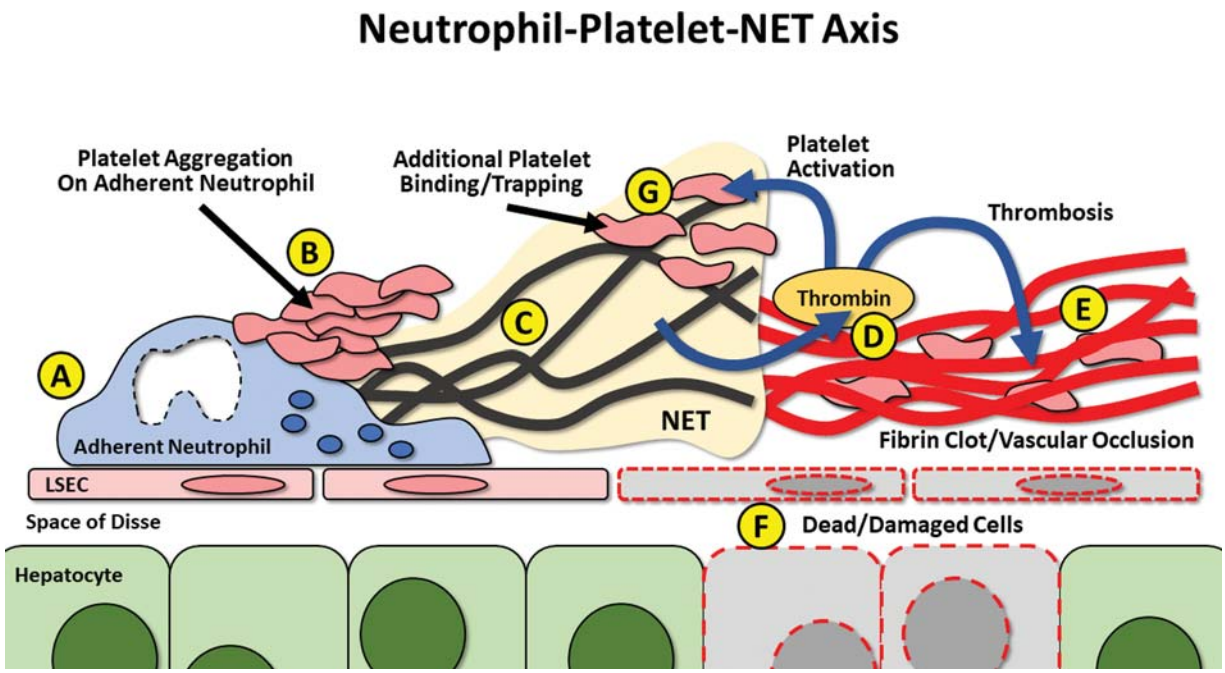

Fig. 1 Schematic of the neutrophil–platelet–NET axis within the liver. Following infectious or sterile inflammatory stimulus, neutrophils are rapidly recruited to, and adhere within, the liver vasculature (**A**). Adherent neutrophils form a platform, supporting integrin-mediated platelet binding and aggregation on the cell surface (**B**). Platelet–neutrophil interactions trigger the release of NETs (**C**). Intravascular NETs, through the actions of multiple components (poly-P, histone), activate coagulation to generate thrombin (**D**) leading to the generation of microvascular thrombi (**E**) within the liver sinusoids. The combined effect of the cytotoxic NET structures and microvascular occlusion leads to LSEC and hepatocyte cell damage and death (**F**). Additionally, the NET serves to bind additional platelets that are activated by proteases on the NET and by thrombin (**G**), amplifying this potentially pathogenic inflammatory response. LSEC, liver sinusoidal endothelial cell; NET, neutrophil extracellular trap.

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amplification of the platelet activation.[33] Additionally, NETs directly activate coagulation within the blood stream, leading to the generation of thrombin and ultimately deposition of fibrin.[32] This process has been attributed to both histone and polyphosphate entities located within the NET matrix as neutralization of either by administration of blocking antibodies prevents coagulation activation. Further contributing to the procoagulant potential of NETs is the DNA scaffold itself. The DNA backbone of NETs possesses a strong negative charge and can serve as a platform for the association and assembly of positively charged coagulation factors,[28] enhancing their activity and efficacy. Moreover, NE, a protein component of NETs, has been shown to facilitate the degradation of tissue factor pathway inhibitor,[34] thereby further enhancing coagulation. Altogether, NETs induce coagulation through a diverse array of overlapping and complementary mechanisms (►**Fig. 2**), representing a potent, multifaceted force linking infection and inflammation with intravascular coagulation. Additionally, it has been suggested that immunothrombosis itself further enhances host immunity, shutting down circulation within some regions of the microvasculature and thereby closing off the conduits used by pathogens to disseminate throughout the body.[35]

Not only does generation of thrombin lead to fibrin clot formation, but generation of thrombin by NETs also leads to amplification of platelet activation through protease-activated receptors (PARs) expressed by platelets (PAR1 and 4 on human platelets, PAR4 and 3 on mouse platelets).[36] Moreover, other neutrophil proteases and NET components have been shown to act on PARs, triggering an alternate signaling pathway leading to further regulation of platelet activation.[37] This feedback loop creates a scenario whereby platelets induce the production of NETs and NETs themselves trigger further activation of platelets, acutely amplifying inflammation, coagulation, and tissue damage. Inhibition of NET generation, or treatment with intravenous DNase to break down NETs, prevents the formation of thrombi and reduces observed tissue damage.[26,32] In addition to local patches of ischemia, this intravascular coagulation within the liver sinusoids drives other pathological responses. Studies using a model of mechanical stretch to activated LSEC, leading to elevated chemokine ligand 1 (CXCL1) expression and increased neutrophil recruitment, identified a linkage between sinusoidal microthrombi and portal hypertension.[38] Moreover, studies conducted in mice deficient in PAD4 or NE (either deficiency results in neutrophils that are unable to produce NETs) highlighted the role of NETs in this process. In animals unable to release NETs, portal hypertension was reduced and less fibrosis within the liver was observed.

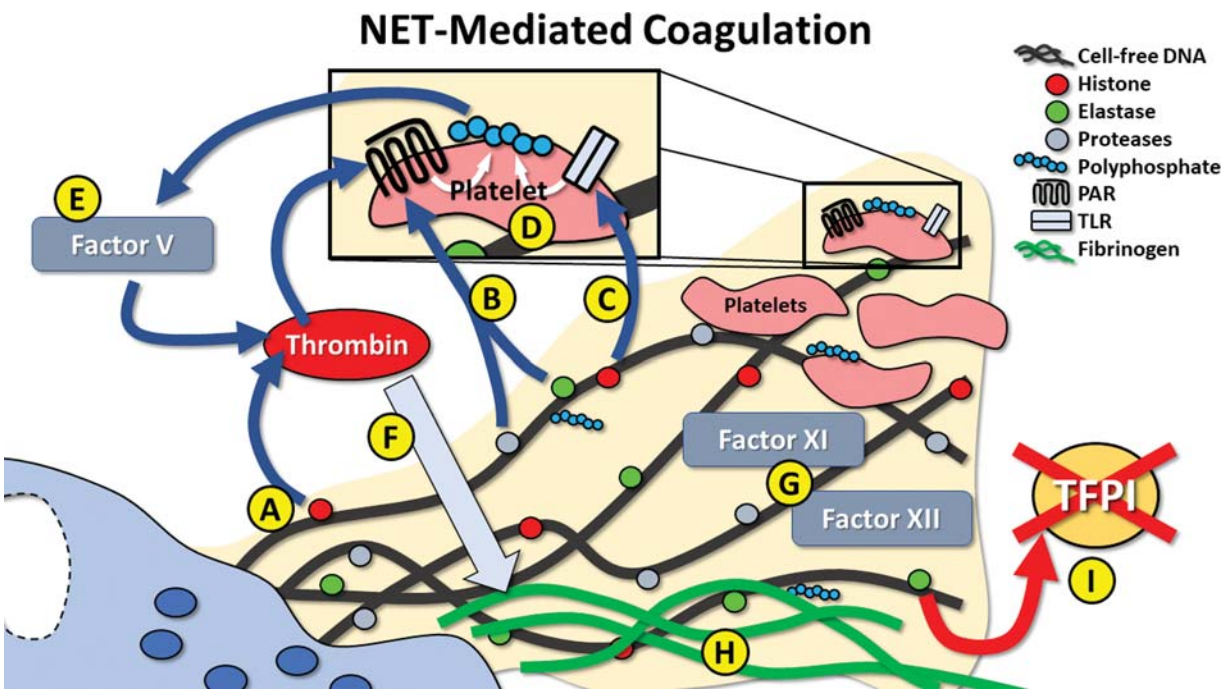

Fig. 2 Schematic of the interplay between NETs and coagulation. NETs represent a multifaceted driver of intravascular coagulation. (**A**) Protein components of NETs such as histones directly activate coagulation to generate thrombin.[31,32] Other components of NETs, such as neutrophil elastase and neutrophil proteases (**B**), activate platelets through PARs,[37] and histones contained in the NET further activate platelets through TLRs (**C**).[33] This platelet activation leads to degranulation and deposition of polyphosphate on both the platelet surface and the NET (**D**). In turn, polyphosphate (**E**), through facilitating the activation of clotting factor V, further drives thrombin activation which positively feeds back on platelet activation through PARs and TLRs.[86,87] Ultimately, thrombin generation culminates in the proteolytic cleavage of fibrinogen (**F**), generating fibrin, the structural basis of thrombi. Additionally, the NET DNA scaffold acts to bind and stabilize other clotting factors, such as factor XI and factor XII (**G**), continuing to drive coagulation.[88] The NET also serves to bind and protect plasma fibrinogen (**H**), preventing its degradation, promoting its conversion to fibrin, and enabling cross-linking, stabilizing the thrombus.[64] Finally, enzymes on the NET, including neutrophil elastase (**I**), have been shown to degrade TFPI, thereby preventing the breakdown of thrombi generated by the NET.[34] NET, neutrophil extracellular trap; PAR, protease activated receptor; TFPI, tissue factor pathway inhibitor; TLR, toll-like receptor.

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Although the linkage between NETs and coagulation in the liver vasculature in animal models is clear, direct clinical evidence (histological data confirming thrombus formation in the microvasculature of the liver) for this hypothesis is limited. Importantly though, in a small randomized controlled trial, patients with cirrhosis who received anticoagulant treatment, specifically enoxaparin, had decreased disease progression and experience reduced occurrence of thrombotic complications compared with cirrhotic patients who did not receive anticoagulation,[39] providing functional, if not histological, support for the hypothesis that inflammation-induced coagulation drives liver disease.

Hence, platelet–neutrophil interactions represent a double-edged sword, triggering the production of intravascular NETs to capture and clear pathogens while potentially amplifying inflammation and collateral damage. Recent work has tried to address the question whether the role of NETs in limiting pathogen dissemination outweighs potential damage to host tissues. In a model of intravenous *Staphylococcus aureus* infection, neither removal of NETs nor inhibition of immunothrombosis impacted bacterial dissemination; however, both treatments limited liver damage and preserved organ function.[40] Although this study only addressed one pathogen in a simple bloodstream bacteremia model, the results suggest in an otherwise healthy individual that NETs are dispensable in host control of infection and instead represent a significant contributor to infection-induced host tissue damage and brings into question the very concept of immunothrombosis as a host-defense mechanism.

Part II: Clinical Perspective

There is substantial experimental evidence for a key role of NETs in the inflammation–coagulation interplay that is suggested to drive liver disease. However, clinical data for this hypothesis are inconclusive and a direct role for NETs in liver disease remains elusive. In this section, clinical evidence for the involvement of NETs in different etiologies and complications of human liver disease will be presented. Moreover, the possible use of NETs as a diagnostic marker and as a target for future therapeutic strategies in liver disease will be explored.

Neutrophil Extracellular Traps in Steatohepatitis

Alcoholic and nonalcoholic steatohepatitis (NASH) remain the main cause of end-stage chronic liver disease worldwide. Although having different triggers, the histopathological stages of disease are similar, specifically steatosis, (acute) hepatitis, fibrosis, and cirrhosis, with the latter predisposing for the development of hepatocellular carcinoma (HCC). Traditionally, treatment is centered around eliminating the trigger through life-style changes, but there remains a need for pathophysiologically targeted treatment options.[41,42] The pathophysiology of acute steatohepatitis is characterized by (sterile) inflammation dominated predominantly by a neutrophil influx into the liver. These neutrophils are thought to cause hepatotoxicity by secreting proinflammatory cytokines and radical oxygen species.[43,44] Histological analyses of 35 human liver biopsies from patients with alcoholic hepatitis showed localization of neutrophils in hepatocyte-apoptotic areas, supporting the hypothesis that neutrophils contribute to liver damage.[45] However, the exact mechanisms by which neutrophils contribute to steatohepatitis are incompletely understood. NETs represent a possible mechanism of disease and could provide a potential new treatment target. This hypothesis is substantiated by a recent study using a mouse model of acute alcoholic hepatitis, where LPS-induced endotoxemia resulted in increased neutrophil accumulation and NET release in the liver (evidenced by immunohistological staining for citrullinated histones), increased liver inflammation, and hepatocyte death in alcohol-exposed mice compared with alcohol-naive mice.[46] In another experimental study, depletion of NE in obese mice resulted in significantly reduced liver inflammation and reduced neutrophil recruitment to adipose tissue in comparison to wild-type obese mice, suggesting that neutrophils, and specifically NE, could contribute to progression of liver inflammation/steatohepatitis.[47] Moreover, a recently published study demonstrated that patients with NASH had elevated plasma levels of markers for NETs.[48] This study compared levels of MPO–DNA complexes, a granular protein component of NETs bound to extracellular DNA, in plasma samples taken prior to hepatectomy for HCC, benign tumor, or liver metastases from patients with or without NASH in the remnant liver and found significantly higher levels of the NET-specific marker in the NASH patients compared with the matched controls. These results suggest a role for NETs in the development and/or the progression of NASH and represent a previously unexplored immune effector mechanism in steatohepatitis that could have important clinical implications. Although exciting, it is important to note that these studies are still in their early stages and these data require further investigation before effective therapies could be developed/trialed.

Neutrophil Extracellular Traps in Autoimmune Liver Disease

Autoimmune liver diseases, such as autoimmune hepatitis, primary sclerosing cholangitis, and primary biliary cholangitis, are trigged in patients with specific genetic predispositions, resulting in a dysregulated immune response to liver autoantigens. Although this immune response involves multiple immune cell types, the early phase of autoimmune liver disease is dominated by neutrophil accumulation.[49] Whether these neutrophils form NETs that could potentially aggravate the course of disease has not yet been studied. Notably, NETs have been extensively studied in other types of autoimmune diseases,[50–52] and have been implicated as a contributor to disease flares. In particular, kidney biopsies from patients with autoimmune small-vessel vasculitis demonstrated NETs near injured glomeruli on immunofluorescence staining for DNA, histones, MPO, and NE.[51] Furthermore, NETs were primarily present in patients with more active disease and were correlated with neutrophil influx. In patients with active small-vessel vasculitis, plasma levels of MPO–DNA complexes were significantly higher than in healthy controls or in patients with disease in remission, suggesting that NETs play a role in active autoimmune disease. Moreover, a recent study described

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elevated plasma levels of MPO–DNA complexes in patients with systemic lupus erythematosus that were associated with severity of disease and, interestingly, with future, but not with current activity of disease.[53] These findings suggest that plasma levels of NETs could potentially aid in the prediction of which patients will need intensified treatment or a change in therapeutic strategy. Liver-specific studies into NETs are currently lacking and may provide a window of opportunity in the prediction and possible treatment of autoimmune liver diseases.

Neutrophil Extracellular Traps in Acute and Acute-on-Chronic Liver Failure

Both acute liver failure (ALF) and acute-on-chronic liver failure (ACLF) are accompanied by a massive release of inflammatory molecules (cytokine storm), often followed by multiorgan failure and death. Currently, it is difficult to stratify risk of progressing to a more severe disease or death in these patients using the existing risk scores.[54,55] Several studies have described the use of cell-free DNA, often referred to as NET marker (discussed in more detail in the diagnostic section below), as a clinical tool to estimate chance of survival in patients with sepsis.[56,57] In accordance with results from studies in septic patients, it was shown that increased levels of cell-free DNA were associated with 30-day mortality in patients with ACLF.[58] Importantly, however, there was no association between levels of the more specific NET marker, MPO–DNA complexes, and mortality in this ACLF cohort ($n = 57$), indicating that massive cell death, and not NETs, might be a prognostic factor for mortality. It should be noted that the plasma levels of MPO–DNA complexes were significantly elevated in patients with ACLF in comparison to healthy controls, suggesting at least the formation of NETs in ACLF. The authors also studied the contribution of NETs to the hemostatic imbalance, which is central in patients with ALF and ACLF, as NETs are known to promote thrombosis at least in experimental models. Markers of NETs were not associated with activation of coagulation and therefore it was concluded that the hemostatic imbalance in these patients was not driven by the formation of NETs. In conclusion, the contribution of NETs to pathological mechanisms of disease or clinical deterioration was not evident from these results, nor was the clinical significance of using NETs as a marker for clinical outcome in ACLF.

Preliminary results from a study by the U.S. ALF Study group showed that patients with ALF or severe acute liver injury from the U.S. ALF Study group registry ($n = 676$) had 6.8-fold higher plasma levels of cell-free DNA and 2.5-fold higher plasma levels of MPO–DNA complexes in comparison to healthy controls. In contrast to patients with ACLF, levels of MPO–DNA complexes, and not of cell-free DNA, were associated with death or (highly urgent) liver transplantation, suggesting that NETs might contribute to disease progression in ALF (von Meijenfeldt FAv (BSc) et al, August 2020, unpublished data). The discrepancy between these two studies might be explained by differences in pathogenesis. Patients with ACLF have chronic liver disease, which might be accompanied by neutrophil dysfunction that impairs the formation of NETs, where ALF patients had good functioning livers prior to the acute onset of inflammation and liver failure. However, it should be noted that the ACLF cohort was relatively small and a limitation of both studies is that NETs were only studied in plasma. It is conceivable that NETs and their link to (micro)thrombosis may remain largely localized to the liver and, as such, a focused study of NETs and thrombi within the liver needs to be initiated.

Neutrophil Extracellular Traps in Ischemia-Reperfusion Injury and Liver Transplantation

More than 20 years ago, platelet–neutrophil interactions were described to drive ischemia-reperfusion injury in liver transplantation.[59] More recent (animal) studies have shown that following ischemia, the reintroduction of oxygen-rich blood leads to an enormous production of reactive oxygen species and damage-associated molecular patterns, which in turn triggers the formation of NETs.[24,60,61] Animal studies have determined that liver ischemia leads to NET formation within the sinusoids, leading to elevated inflammation and liver injury. Experimental conditions that reduce or remove NETs (PAD4 deficiency, intravenous DNase treatment) reduced the quantity of NETs following ischemic challenge and were associated with a concomitant reduction in inflammation and tissue injury.[24]

To establish whether NET formation occurs following ischemia-reperfusion in human liver transplantation, we have measured markers for NETs in plasma of patients undergoing liver transplantation, and hypothesized that levels would peak after reperfusion.[62] Indeed, levels of cell-free DNA were significantly elevated after reperfusion, peaked at the end of transplantation, and interestingly, were associated with activation of coagulation. Levels of the more specific NET marker, MPO–DNA complexes, were already significantly elevated at the start of transplantation in comparison with healthy controls, which might indicate NET formation in patients with end-stage liver disease. MPO–DNA complex levels peaked in the anhepatic phase, which could be explained by increased formation of NETs or by decreased clearance of MPO–DNA complexes by the liver, and remained elevated until the end of transplantation, but were not associated with activation of coagulation. The different distribution of cell-free DNA and MPO–DNA complexes during liver transplantation suggests that cell-free DNA was not derived from NETs, but more likely from dead hepatocytes or other injured cells. In addition to plasma, we obtained recipient liver biopsies 30 minutes after reperfusion, which showed a distribution of NE in the liver tissue which was suggestive for the presence of NETs. The difference between systemically and locally measured NETs underlines the importance of using standardized and validated assays that are specific for NETs and cautious interpretation of results. Since various animal studies have shown the protective effect of NET inhibitors on ischemia-reperfusion-associated (micro)perfusion deficits and organ damage,[24,60,61,63] future research could explore the use of NET inhibitors in reducing ischemia-reperfusion injury in a clinical setting.

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Neutrophil Extracellular Traps in Thrombotic Complications of Liver Disease

Multiple preclinical studies have shown that NETs promote thrombus formation via various mechanisms and that by blocking NET formation thrombosis is effectively inhibited.[31,64,65] This is especially of interest in the context of the liver, an organ that plays a central role in thrombosis and hemostasis and, as a consequence, plays a central role in both the bleeding and the thrombotic complications that are commonly observed in patients with liver disease. In clinical practice, although much of the emphasis still lies on the presumed bleeding tendency of patients with liver disease, there is substantial evidence pointing at a rebalanced hemostasis in even the very ill patients.[66,67] As such, clinical attention might need to be shifted toward the prothrombotic tendency, substantiated by the increased risk of deep venous thrombosis within cirrhotic patients.[68] In liver surgery and transplantation, thrombotic complications are feared both before, in the form of portal vein thrombosis (PVT), and after, as for example hepatic artery thrombosis, conditions that are associated with increased morbidity and mortality.[69] To our knowledge, clinical studies on NETs in liver-specific thrombotic complications are scarce, but there is comprehensive clinical evidence for a critical role for NETs in thrombosis. Multiple clinical studies have shown that increased plasma levels of the nonspecific marker cell-free DNA is associated with the severity of thrombotic diseases, namely myocardial infarction, stroke, and venous thromboembolism.[70] Furthermore, patients with high levels of nucleosomes (histone–DNA complexes) had a threefold higher risk of deep venous thrombosis after adjustments for other known risk factors.[71] These studies suggest that NETs could be a risk factor for thrombosis and importantly, that markers of NETs could be used in a risk score for prediction of (venous) thromboembolic events, though controlled prospective clinical trials would be required to fully evaluate the value of measuring NET levels as a clinical predictor.

The involvement of NETs in thrombus formation is supported by histological data generated from human thrombus material. For instance, the composition of 81 human thrombi obtained during percutaneous coronary intervention for myocardial infarction showed substantial numbers of neutrophils in the thrombus material, cellular sources which could potentially form NETs.[72] Furthermore, immunohistological analyses performed on 68 human ischemic stroke thrombi demonstrated the presence of NETs in the thrombus material. All thrombi were positive on immunostaining for both citrullinated histones and NE and showed colocalization of DNA, neutrophils, and histones by immunofluorescence staining.[73] Moreover, ex vivo clot lysis assays of the human ischemic stroke thrombi with either tissue plasminogen activator (tPA) alone or tPA and DNase resulted in a significantly reduced thrombus weight in the DNAse group, suggesting that NETs not only contribute to thrombus generation but may also contribute to clot stability, and that DNase could potentially be used to prevent or treat thrombosis in these conditions.

PVT is a common complication in patients with liver disease and presents with a pathophysiology that is incompletely understood. Moreover, PVTs are difficult to treat, partly because anticoagulant therapy is only effective in a proportion of patients.[74] A recent retrospective study into risk factors for PVT in patients with HCC reported significantly higher plasma levels of cell-free DNA, nucleosomes, and NE in patients with PVT compared with patients without PVT.[75] Although the NET markers had significant odds ratios for assessing thrombotic risk in the univariable logistic regression analysis, these results were not confirmed with multivariable analyses, implying, in this study, that NETs were not an independent risk factor for the development of PVT in patients with HCC.

Overall, clinical data on NETs in liver-specific thrombotic complications are limited, and the role of NETs in prediction models for PVT is questionable. However, the prominent role for NETs in thrombosis and the need for novel treatment options make studies into liver-specific thrombotic complications of critical importance.

Neutrophil Extracellular Traps in Liver Cancer

The microenvironment of tumors often contains neutrophils, which have been attributed both to pro- and antitumorigenic functions. This pro/antitumor role is thought to be dependent on the type of neutrophil that is involved. N1 neutrophils activate the immune system and induce cytotoxicity of cancer cells, whereas N2 neutrophils promote progression of tumors by secreting growth factors and stimulating angiogenesis.[76] Likewise, NETs have been implicated in both inhibition and progression of cancer, although their exact contribution remains uncertain. In particular, NETs have been shown to help promote tumor metastasis to the liver. Studies have shown that NETs present within the liver vasculature enhanced trapping of circulating metastatic tumor cells, causing these cells to lodge in the liver sinusoids, establishing new tumors.[77] In these studies, NETs were associated with increased local and distant metastases, and blockage of NETs resulted in a decreased extent of metastasis.[78] Furthermore, once adherent, metastatic cells have been shown to internalize fragments of NETs, driving TLR9 activation and leading to NFκB and COX2-mediated signaling, ultimately resulting in increased cell survival and enhanced tumor establishment.[79] It is argued that this inflammation/NET-mediated tumor cell trapping may partly explain postsurgical metastasis of colon carcinoma to the liver. Resection of the primary colon tumor is believed to release PAMPs such as LPS into the circulation, triggering NET production in the liver and facilitating the trapping of circulating tumor cells that may have been liberated by the surgical process.

In accordance with these results, a clinical study showed that patients undergoing liver resection for colorectal liver metastasis that had increased plasma levels of MPO–DNA complexes postoperatively had a four times higher risk for recurrence of metastases compared with patients with low levels of MPO–DNA complexes.[80] These results indicate that NETs could promote metastases and targeting NETs could reduce recurrence/progression of cancer. Importantly, as NETs have been implicated in thrombotic disease, it is

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conceivable that NETs contribute to cancer-associated thrombosis, making it an even more interesting therapeutic target.

Diagnostic Modalities and Future Therapeutic Strategies

The results of clinical studies on the prognostic utility of NETs in the treatment of liver disease are often contradicting and clinical data are scarce. Given these often-conflicting findings, it must be noted that the data regarding NETs and liver disease are only as reliable as the technique used to measure the NETs themselves. This is an area of much debate, and the establishment of a universal standard for the measurement of patient NET levels is warranted. Currently, several different diagnostic tools and quantification of multiple different markers have been used (►**Table 1**) with limited effort to reconcile the various platforms.

One of the most commonly used approaches involves enzyme-linked immunosorbent assays (ELISAs) for the quantification of a protein component of NETs (e.g., histone, NE, and MPO). ELISAs are sensitive, easy to use, rapid, and are adaptable to possible implementation in (acute) clinical care. Despite these clear advantages, ELISAs are only able to measure derivatives of or break down products of NETs in fluids or plasma, and often the specific cellular origin of the DNA remains uncertain. The specificity of this approach can be greatly enhanced by measuring linked complexes of proteins. For example, assays that capture histone but detect NE (two components of NETs linked by the DNA backbone and that should not exist as a complex outside of the NET structure) can be used to specifically differentiate between NETs and other markers associated with total cell-free DNA.

Other techniques can be used to directly quantify circulating cell-free DNA. Through the application of polymerase chain reaction (PCR) techniques, trace amounts of DNA can be amplified and quantified from a variety of patient samples. This approach can allow the origin (host vs. pathogen) and length of DNA molecules to be identified. Although often more sensitive than ELISA-based techniques, the difficulty in differentiation between DNA released from damaged tissue and de facto NETs makes the use of PCR controversial in clinical assessment of NETs in patients.

Immunohistological staining of tissue sections/biopsies with antibodies directed against citrullinated histones, NE, and MPO can specifically identify NETs, allowing for identification of these structures in human tissue. Though definitive, immunostaining is time-consuming, expensive, and is sensitive to variation between laboratories. Moreover, immunostaining requires patient tissues and thus is more invasive than assessment of patient plasma. Additionally, if there is localized heterogeneity of NET distribution (patches of NETs interspersed with areas of healthy tissue), it is possible that a small biopsy may not provide a representative assessment of the overall NET content of a given tissue. Despite these limitations, immunofluorescence staining offers the possibility to colocalize components of NETs with specific tissue pathologies and structures. For example, addition of other labeled antibodies can allow for counter-staining of blood vessel walls, intravascular leukocytes (KCs), and viral pathogens in conjunction with NETs within the liver microenvironment. Importantly, immunostaining can be adapted to the technique of intravital imaging of animal models of liver disease, providing insight into living tissues and relating NETs to specific cell behaviors and responses. Furthermore, through the implementation of technological advances, such as

Table 1 Comparison of various diagnostic techniques for the quantification and characterization of NETs within a patient sample

Assay	Advantages	Limitations
Single molecule ELISA (i.e., histone, neutrophil elastase)	• Quick, easy • Sensitive • Commercially available	Unable to determine if the detected molecules are associated with a NET or from another source (i.e., neutrophil degranulation, cell death)
ELISA for molecular complexes (i.e., histone–elastase, histone–MPO)	• Quick, reproducible • Sensitive • Can differentiate molecules on a NET from other sources	Typically, not commercially available requiring kits to be "home-made" using parts from other assays Samples are largely limited to patient fluids (blood, sputum, etc.)
Cell-free DNA by PCR	• Very sensitive • Low cost • Can differentiate between host DNA and pathogen DNA (i.e., biofilms)	Unable to differentiate between NETs and DNA released from cell lysis, a critical problem when studying conditions such as sepsis where extensive host cell death is observed
Immunofluorescence	• Determine localization of NETs relative to cells, tissue structure, damage • Colocalize multiple molecules/cells • Can adapt to intravital microscopy of animal models of disease	Slow, expensive, and highly variable between laboratories Requires interpretation of images by experienced researchers Requires tissue sections/biopsy from the patient (invasive)
Electron microscopy	• Gold standard • Able to identify the NET DNA backbone structure, identifying the pearls-on-a-string appearance of nucleosomes along decondensed DNA	Slow, requires specialized equipment and expertise Unable to study NETs in a dynamic fashion Requires tissue biopsy from the patient (invasive)

Abbreviations: ELISA, enzyme-linked immunosorbent assay; MPO, myeloperoxidase; NET, neutrophil extracellular trap; PCR, polymerase chain reaction.

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machine learning, we can better develop an understanding of how NETs are associated with tissue structures, immune cell populations, local pathologies, and specific disease processes in a more mechanistic and predictive fashion.[81]

Arguably, the gold standard for detection and characterization of NETs is electron microscopy. It was through this approach that NETs were first identified, directly visualizing the DNA scaffold, the presence of nucleosomes, and the association of the NET with pathogens.[18] Coupled to immunogold labeling for other NET components (NE), this approach can efficiently characterize the composition of the NET, providing unequivocal evidence of the structure and function of these immune effector molecules. Despite these high-resolution data, electron microscopy is fraught with similar limitations to those associated with immunofluorescence. It is time-consuming, expensive, and has the requirement for invasive tissue biopsies to visualize the association between NETs and organ pathology. Although an important technique for NET research, these limitations mean electron microscopy has seen limited use as a front-line diagnostic tool in the study of NETs and clinical disease.

Regardless of the technique used to measure NETs, the need for standardization of measures with validated assays must be stressed. These standards must be available to all investigators and must be easy to use and reliable. In many ways this highlights the fundamental problem with clinical assessment of NET levels in patients—the lack of a single gold-standard assay. This critical limitation is the focus of working groups of experts who are currently drafting unified recommendations for the detection and quantification of NETs within the clinical setting. Establishment and adoption of these universal recommendations will allow for the generation of reliable and reproducible clinical datasets that will aid in our understanding of the role of NETs in the development and progression of liver disease.

As our knowledge of NETs in disease advances, we will likely encounter situations where NETs may present as an attractive therapeutic target. The fragile balance of the hemostatic system in patients with liver disease has resulted in a restrictive (and cautious) use of anticoagulants in patients with liver disease. Despite substantial evidence that liver patients are at increased risk for (micro)thrombosis, anticoagulant treatment is often withheld. Importantly, data on the pharmacokinetics, dynamics, and dosing of anticoagulation in this specific patient group are lacking and conventional anticoagulants fail to differentiate between inflammation-induced coagulopathy and hemostasis.[82] In this regard, NETs provide an attractive new potential therapeutic target to prevent thrombosis that is able to uncouple inflammation-driven thrombosis from hemostasis. Numerous animal studies have demonstrated that inhibition of NETs can prevent (micro)vascular thrombosis and can reduce overall liver injury.[32,38] By targeting NETs, it may be possible to block pathogenic thrombosis without elevating the risk of bleeding in the patient, the primary reason for the restraint in prescribing conventional anticoagulation for patients with liver disease.

Although disruption of NETs may allow for the separation of thrombotic risk from hemostasis, this approach raises the concern of limiting the immune function of neutrophils, potentially leading to an increased risk of infection or sepsis. Experimental studies have shown that local colonization and persistence of the bacterial infection were enhanced, and bacterial dissemination was increased by blocking NET formation in animal models of bacterial endocarditis and pneumonia.[83,84] However, in other studies, blocking NET formation in animal models of sepsis reduced liver injury, improved liver (micro)perfusion and function, and did not result in increased bacterial dissemination.[28,40,85] In these models of sepsis, it was suggested that the loss of immune protection provided by NETs was offset by a gain of function by other immune cells in the liver. It has been shown that mice in which NETs were degraded (DNase treatment) had preserved immune function, with KCs and splenic macrophages compensating for the loss of immunoprotection attributed to NETs. Future research into NETs in the treatment of various types of (complications) of liver disease is greatly encouraged, where the presumed benefits should be carefully weighed against the risks.

Summary and Conclusion

NETs are a unique feature of the immune system that form a web-like structure to trap and remove pathogens and activate other immune cells. In addition to their immune function, NETs have been shown to amplify inflammation, directly inflict host tissue damage, and activate coagulation further amplifying collateral damage to surrounding tissue through microvascular occlusion and ischemic injury. In liver disease, where neutrophil accumulation and activation of coagulation are central mechanisms of pathogenesis, understanding the role and contribution of NETs to disease progression is critical. Increasing evidence suggests that platelet–neutrophil–NET interactions drive liver disease and that NETs could be a potential new therapeutic target in the treatment of liver disease and its complications. Despite this clear linkage, specific clinical evidence is sparse. Given the therapeutic potential to target NETs within the context of liver disease, these clinical studies are essential to building a mechanistic knowledge of how NETs initiate, support progression of, and lead to significant complication of liver disease in the patient.

Conflict of Interest
The authors declare no competing interests.

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